The Development of the Nervous System in Chick Embryos, studied by Electron Microscopy

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WITH SEVEN PLATES

An electron microscope study of developing nervous tissue has been made in the chick embryo, correlating the results with those obtained by other techniques. I have been especially concerned with (1) the relationship between ectoderm and mesoderm cells at the time of neural induction, and (2) the formation of the Nissl substance.

MATERIAL AND METHODS

Tissue from 73 chick embryos incubated for periods between 1 and 13 days has been studied. Before fixation each specimen was staged according to the normal table of Hamburger & Hamilton (1951). The tissue was fixed either in osmium tetroxide, or in potassium permanganate (Luft, 1956). In either case the pH was buffered to 7.4 with sodium veronal (Palade, 1952) and fixation was at 0° C. In the early stages the whole embryo was fixed. In the later stages the nerve-cord was dissected out partially before fixation, and completely afterwards. Dehydration was with alcohols, and the specimens were then embedded either in the epoxide resin ‘Araldite’ (Glauert, Rogers, & Glauert, 1956) or in methacrylate. Sections were examined in a Siemens Elmiskop 1B electron microscope.

Twenty-four specimens have also been studied by light microscopy. Twelve of these were stained with silver (Bielschowsky’s method) and twelve were selectively stained for Nissl substance with dahlia, cresyl violet, or neutral red.

Quantitative analysis of the cellular constituents

Twenty-five specimens at seven different stages were analysed by an adaptation of Chalkley’s method (1943) as used in light microscopy. Montages were taken, each consisting of about twelve electron micrographs with magnifications

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of either 35,000 or 70,000 after printing. A piece of transparent paper the same size as the photographs was marked into a grid by two sets of parallel lines at right angles to one another (see Text-fig. 1). The grid was placed over each photograph and the points where the grid lines intersected were used for sampling. There were 180 such points on the grid. Counts were made of the number of sampling points which fell on the following structures in each photograph: mitochondria, endoplasmic reticulum, cytoplasm (other than the constituents already mentioned), nucleus. The process was repeated for each photograph in the montage, care being taken where two photographs overlapped not to count the same area twice. The number of points falling on the mitochondria, and of those falling on the endoplasmic reticulum (see Table 1) were then expressed for each embryo as a percentage of those falling on the 'total cytoplasm' ('total cytoplasm' has been considered as the sum of the following structures: endoplasmic reticulum, mitochondria, intra-cellular yolk drops, granules of all kinds, and the spaces between these structures. Nuclei, nuclear and cell membranes and inter-cellular material have not been included). The individual percentages for several embryos were then combined to give the mean percentage for each stage. Each
point shown on the graphs (Text-fig. 2) is thus derived from between about 3,000 and 6,000 readings of the sample points on the grid. The percentages of sampling points may be taken to represent the percentage amounts of mitochondrial or endoplasmic reticular substance present in the cytoplasm.

### Table 1

**Numbers of points falling on different components of the cytoplasm, according to an adaptation of the method of Chalkley for estimating areas.**

*See Text-figs. 1 and 2*

<table>
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<th>Stage</th>
<th>Estimated mean age in hours</th>
<th>Specimen</th>
<th>Endoplasmic reticulum</th>
<th>Mitochondria</th>
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**Stages examined by electron microscopy**

1. **The long primitive-streak stage.** This stage, as defined by Waddington (1932) and Abercrombie (1950), is later than stage 3½ of Hamburger & Hamilton (1951) and earlier than their stage 4 (Text-fig. 3 A, D).

2. **The head-process stages.** Stages 4 and 5 of Hamburger & Hamilton (1951). Stage 4 is often called the definitive primitive-streak stage and many authors have considered it to be equivalent to the long primitive-streak stage. Fig. 4 of Hamburger & Hamilton (1951) shows, however, that some of the head-process mesoderm already lies anterior to the primitive streak in stage 4 (see also Text-fig. 3 B, C, E).
3. Stages 10–14 of Hamburger & Hamilton (1951). These stages were reached after about 40 to 48 hours’ incubation (Text-fig. 3f).

4. Stages 16–22 of Hamburger & Hamilton (1951). These stages were reached after 2½–4 days’ incubation (Text-fig. 3g).

5. Stages 31–39 of Hamburger & Hamilton (1951). These stages were reached after 7–13 days’ incubation (Text-fig. 3h).

RESULTS

Early stages with special reference to neural induction

The long primitive-streak stage

The ectoderm lying anterior to the primitive streak is a pseudostratified epithelium about 30 to 40 µ in depth and may have as many as 3 or 4 nuclei between dorsal and ventral sides. It is part of the presumptive neural plate (Pasteels, 1958).
1937), but apart from a few scattered cells it is not yet underlain by mesoderm (Text-fig. 3d). It contains intra-cellular yolk droplets (Bellairs, 1958).

Plate 1, fig. 1 is a low-power electron micrograph of a section through the presumptive neural plate ectoderm just anterior to the primitive streak. Two endodermal cells are at the bottom of the picture; they are not in contact with the ectoderm. The section passes through the whole depth of the ectoderm and one cell can be seen to stretch from the top to the bottom of the epithelium. The ectodermal cells are separated by an intercellular matrix about 100–150 Å wide in most parts, although there are often small triangular or diamond-shaped spaces where the borders of three or more cells meet. At the dorsal surface small filamentous cytoplasmic strands covered by cell membrane often project from the free edge of the cells.

At low electron magnifications a dense line about 250 Å wide lies parallel to the cell membrane at the ventral surface of the ectoderm. It is separated from the cell membrane by a lighter zone which is about 250 Å wide. This dense line
DEVELOPING NERVOUS TISSUE

is also found along the ventral border of the neural plate, or neural tube, in all the following stages (Plate 1, fig. 5, d.l.). At low electron magnifications it sometimes gives the impression of a membrane lying parallel with the cell membrane (Plate 1, fig. 5, d.l.). At higher magnifications, however, both the dense line and the light zone can be seen to consist of a fine granular material (Plate 1, fig. 3, d.l. and l.z.). This fine granular material differs from the cell membrane in appearance.

Between the ectoderm (or neural tube) and the mesoderm can be seen 'free material' (Plate 1, fig. 5, f.m.), some of which appears to be attached to the 'dense line'. At higher magnifications these can be resolved into granules, each about 100–150 Å in diameter (Plate 1, fig. 4, gran.). These granules, like similar ones in the cytoplasm, cannot be seen after fixation in potassium permanganate. After fixation in osmic acid they are found so intimately associated with the finer granular material of the 'dense line' that it is difficult to resolve the 'dense line' at high electron magnifications (Plate 1, fig. 4).

Most of the cells at the primitive-streak stage appear to be six- or eight-sided in section, their longest axis being usually, though not invariably, oriented dorso-ventrally (Plate 1, fig. 1). In all stages examined the nuclei are oval with two membranes which are smooth in specimens fixed in osmium tetroxide, although they are often wrinkled after fixing in potassium permanganate. Nucleoli can be seen in the large nuclei, i.e. in those which have probably been cut through the middle.

Three main types of yolk drop, including all varieties of Type 'A', are present (Plate 1, fig. 1) at the primitive-streak stage. These have been described previously (Bellairs, 1958). The sectioned mitochondria are usually oval in shape, the length seldom being greater than three times the width; their cristae are often disorganized. Both granular (Plate 1, fig. 2, g.e.r.) and agranular membranes of the endoplasmic reticulum (as described by Palay & Palade, 1955) are present, but usually have round or oval outlines in the sections. Occasionally the non-granular type is arranged in the way which many workers (e.g. Sjöstrand, 1956) have considered to be characteristic of the Golgi apparatus. Granules of about 100 Å or more in diameter (Plate 1, fig. 2, gran.) are scanty compared with the stages of 48 hours and later (see Bellairs, 1958).

Stages 4 and 5

The ectoderm is thicker at this stage, being between about 40 and 50 µ, and it seems probable that this is because more cells are present. Some cells still appear to extend through the whole thickness of the epithelium. The presumptive neural-plate region which is anterior to the primitive streak (Pasteels, 1937; Spratt, 1952) lies closely about the head-process mesoderm (Text-fig. 3E).

The principal difference between these stages and the previous one is that the cells have become more elongated, especially in stage 5. The three main types of yolk drop are still present (Plate 2, fig. 6 shows two types) although type B (fatty)
tends to be most frequent at the ependymal edge. The membranes of the endoplasmic reticulum (see Text-fig. 2) usually have circular or oval outlines (Plate 2, fig. 8 e.r.), which suggests that they are perhaps in the form of vesicles; but in certain especially elongated cells they appear as long pairs of membranes (Plate 2, fig. 7, e.r.), which indicates that they may also be in the form of tubules or sheets.

The free edge of the lowermost cells of the neural plate is covered, as in the earlier stage, with a fine granular substance, which resembles the intercellular matrix. It is in fact considered to be the basement membrane (see Discussion). Where the cells of the head-process mesoderm lie closely beneath the ectoderm this granular substance has always (10 specimens) been found to be pressed between them (Plate 2, fig. 8, i.c.m.). It varies in width, even in the same section, and may be as little as 200 Å or as much as 1,000 Å. Particles are present in this material (Plate 2, fig. 9, gr.) which resemble some of the cytoplasmic granules, as well as those of the ‘free material’ (see above), in the following respects: size (100–150 Å); electron density after fixation in osmium tetroxide; arrangement, which may be single or in the form of a ‘rosette’ (Plate 2, fig. 9, gr.); and in being visible after fixation in osmium tetroxide but not after fixation in potassium permanganate.

The mesodermal cells possess the same components as the ectodermal cells; they are, however, more irregular in outline, as is well known to light microscopists. Their nuclei are larger compared with the area of the cytoplasm than those of the ectoderm. It follows that the nuclei of the mesodermal cells often lie close to the cell membrane and therefore close to the ectoderm (Plate 2, figs. 6, 8, n.m.).

Later stages with special reference to Nissl substance and neurofibrillae

Stages 10–14

By stage 10 the neural plate has become converted into a tube along most of its length. It consists of two layers, the ependyma, which lines the neural canal and contains a large number of mitotic cells, and the mantle layer (Text-fig. 3F). At about 48 hours a few neuroblasts can be distinguished in the ventro-lateral part of the tube in silver-stained specimens; this region of the tube is about six cells deep. In the floor of the tube there is only a single layer of cells. Detailed descriptions of the developing neuroblasts at these stages are given by Cajal (1890), Tello (1923), and Hughes (1955).

All the cells are elongated with their long axes radiating out from the ependyma to the peripheral edge of the tube. Occasionally they extend as much as half-way across the wall of the tube. Some cells appear to have a short process extending out from them (Plate 4, fig. 14, c.p.). In some cases this may be a portion of the cell which has been sectioned obliquely. In others, however, the ‘process’ is characterized by possessing several paired membranes of the endoplasmic reticulum running along it (Plate 4, fig. 14, m.e.r.). These membranes of
the endoplasmic reticulum are packed together only in the 'processes' of these cells and seem to be more spread out in the rest of the cytoplasm. It is suggested, therefore, that the 'processes' are true ones and that the cells concerned are probably developing neuroblasts.

The lumen of the tube contains a granular substance which has probably been precipitated by fixation (Plate 3, fig. 10, l). The cells are separated from one another only by an intercellular matrix about 150 A thick.

The types of intra-cellular yolk drops have been described previously and it has been shown that their structure changes as development of the embryo proceeds (Bellairs, 1958). Those which have been classified as 'type A' drops are seldom present at this stage and have only been seen in their final form, that is when they consist entirely of 'circular bodies'. 'Complex type' yolk drops are even rarer. The 'type B' yolk drops, which are highly electron-opaque after fixation in osmic acid, are plentiful. Although they are found in all parts of the neural tube they tend to be commonest in the ependymal region (Plate 3, fig. 10, B).

The mitochondria are usually seen in section as round or oval bodies seldom longer than three times their width (Plate 1, fig. 5, m). The small cytoplasmic particles are more numerous at this stage than in the preceding ones (see Bellairs, 1958). Neurofilaments (see below) have not been seen at this stage. The proportion of both endoplasmic reticulum and mitochondria present in the cytoplasm is shown in Text-fig. 2 and Table 1.

**Stages 16–22**

By stage 16 the neural tube has become closed dorsally along its whole length. In silver preparations neuroblasts can be seen in the latero-ventral parts of the tube and spinal nerves have developed. By stage 22, when more spinal nerves are visible, regions of 'grey' and 'white' matter can be recognized (Text-fig. 3g). Nissl substance cannot be seen in embryos of this stage stained with basic dyes. During this period (stages 16–22) some of the ependymal cells at the two sides of the neural tube begin to come into contact with one another in the midline, thus reducing the size of the lumen. For full descriptions of these stages see Cajal (1890) and Tello (1923).

In these and the following stages I have been concerned mainly with the ventral part of the neural tube in the anterior trunk and neck. The cells are elongated in a medio-lateral direction. They are broadest in the region of the nucleus, and in most of the sections examined their is little space between the nuclear and cellular membranes at the sides of the cell (Plate 5, figs. 19, 20, n.m. and c.m.). In some cells the distal end can be seen as an elongated process extending out towards the edge of the neural tube (Plate 5, fig. 19). Typically, where a process passes between the wide nucleated parts of two other cells it is narrower from side to side, as if it has become constricted, whereas farther laterally it is often wider as if it has been able to swell out again. Similar, though shorter, processes
have also been seen on the proximal sides of these cells. It is thought that these are genuine processes rather than parts of the cell which have been sectioned in an unusual manner, because they are not only rich in paired membranes of endoplasmic reticulum (see previous stage) but elongated sections of cells are more common in this stage than in earlier ones.

Gaps at the place where several cells meet are rare, although they are present in the 'white matter' of stage 22. In the earliest of these stages (stage 16) cells are usually separated by an intercellular matrix between 100 and 150 Å wide. By stage 21, however, the intercellular matrix has been reduced in small patches between adjacent cells to less than 50 Å wide (Plate 3, figs. 12, 13). Small villous-like processes are often packed tightly between adjacent cells (Plate 5, fig. 20). A similar condition occurs in places where the two sides of the ependyma have come together in the midline (Plate 3, fig. 11).

'Type B' yolk drops are scarce and small compared with the previous stages and with the adjacent mesoderm. No 'type A' yolk drops or 'complex' yolk drops have ever been seen in the neural tube at this stage.

The shape and appearance of the mitochondria seem to vary with the specimen, and possibly also with the fixative as noted by de Lorenzo (1957). In some embryos, especially those fixed in potassium permanganate, the mitochondria are circular or oval in section, and their length is seldom more than twice or three times their width. They have a vacuolated appearance which suggests that their cristae have been damaged. In other specimens, especially those fixed in osmic acid, longer sections of mitochondria are also seen, and in these the cristae are usually intact.

By this stage of development the proportion of endoplasmic reticulum to total cytoplasm has increased (see Text-fig. 2). The endoplasmic reticulum is most dense in the processes of the cells (Plate 5, fig. 18) where it is in two forms: (1) pairs of membranes about 150 to 200 Å apart (Plate 5, fig. 18, m.e.r.), and (2) circles or ovals with an internal diameter of about 250 Å (Plate 5, fig. 18, c.e.r.). It is suggested that these are longitudinal and transverse sections of tubules running along the cell processes. Mitochondria (Plate 5, fig. 18, m.) are also present in the cell processes. Neurofilaments, each about 100 Å in width, are present at this stage (Plate 5, fig. 21, n.f.).

In many cells the membranes of the endoplasmic reticulum are often circular or oval in outline (Plate 3, fig. 11, e.r.). In specimens fixed in osmic acid they are mainly of the granular type (Plate 4, fig. 15, e.r.). Some cells near the ependyma possess considerable amounts of endoplasmic reticulum in the form of paired membranes (Plate 3, fig. 11, m.e.r.); these may be cells which are developing into neuroblasts. In many of the sections of ependymal cells, however, both endoplasmic reticulum and mitochondria are scanty (Plate 3, fig. 11, ep.).

Stages 31−39

Dorsal and ventral horns are recognizable in the 'grey matter' from the 7th
day, and glial cells can be seen in the ‘white matter’ (Hughes, 1955). During the following days the nerve-cord becomes larger in transverse section; there appear to be more cells present but individual neuroblasts are also bigger. In specimens stained with basic dyes, Nissl substance can be recognized in a few cells on the 7th day, but is more conspicuous by the 10th day. During this period there is a change in shape of the lumen from a longitudinal slit to an almost square or round shape (Text-fig. 3H). This is accompanied by the development of the dorsal and ventral fissures. For a description of these stages, see Cajal (1890) and Hughes (1955). For an account of the development of the spongioblasts see Bensted, Dobbing, Morgan, Reid, & Payling Wright (1957).

Spongioblasts and glial cells. Although the present paper is not directly concerned with the development of the glial cells an attempt has been made to identify them and to distinguish them from neuroblasts. In the ‘white matter’ the glial cells can be identified with confidence because no neuroblast cell-bodies are present in this region (Plate 7, fig. 28, n.g.). These glial cells are characterized by the fact that they contain very little endoplasmic reticulum compared with the neuroblasts (see below). It is not possible, however, to use this fact for distinguishing spongioblasts and glial cells in the ‘grey matter’, for there are at least three types of cells in that region which have a similar appearance. They are spongioblasts, undifferentiated cells, and partially differentiated neuroblasts.

Neuroblasts. The neuroblasts are characterized by their long axons, which in favourable sections can be seen extending out from the cell-body, and by the presence of large amounts of endoplasmic reticulum in the cell-body (see below). Axons. At least two kinds of filamentous structure appear to run along the axons.

1. Neurofilaments (axon filaments). These structures are about 100 Å in diameter. They have been followed for lengths of up to 2 μ and it seems possible that they may extend the whole length of the axon. Although they are mostly oriented along the axon they frequently cross one another (Plate 4, fig. 16, n.f.).

2. Tubes of endoplasmic reticulum. In longitudinal sections of axons, paired membranes can be seen running lengthwise as if tubes have been cut longitudinally (Plate 6, fig. 26). In every transverse section of an axon examined, membranes of endoplasmic reticulum can be seen in the form of circles or ovals as if tubes of the endoplasmic reticulum have been cut across (Plate 6, fig. 25). In both the longitudinal and transverse sections apparently homogeneous material is enclosed in the tubes. In some regions, however, the tubes appear to be swollen in places (Plate 6, fig. 26, sw.). The swollen regions are usually circular or oval. The walls of the endoplasmic reticulum tubes are about 75 Å wide. In the non-swollen regions the total width of the tubes is usually about 300 Å, but in the swollen region it may be as much as \( \frac{1}{2} \) μ.

In addition to the neurofilaments and the tubes of endoplasmic reticulum, dark wavy bands, each about 150–300 Å in diameter, can often be seen running along the axon after fixation in osmium tetroxide. The number of these bands is
variable, but about five can be seen in Plate 6, fig. 23, d.b. It will be suggested
that they are formed by the clumping together of neurofilaments, perhaps as a
result of over-fixation. In the same axon, circular or oval sections of endoplas-
mic reticulum can be seen (Plate 6, figs. 22, 23, 24). They are between $\frac{1}{4}$ and $\frac{3}{4}$ \(\mu\) in diameter and are strung out in rows along the axons. Sometimes they appear
to be connected by a narrow stalk (arrows, Plate 6, fig. 24). It is suggested that
these may be the swollen regions of the tubes of endoplasmic reticulum and that
they appear in these sections as discrete vesicles because the plane of section has
not passed sagittally through the tube.

The cristae of the mitochondria found in the axons are often oriented longi-
tudinally in the way described by Palay (1956) for adult axons (Plate 6,
fig. 22, m.). Furthermore, the cristae seldom appear to be disrupted at this stage
and the mitochondria as a whole are more electron-opaque than those in the
younger specimens.

The cell-body. In the 'grey matter' the cells lie close to one another, although
large gaps have occasionally been seen (Plate 7, fig. 27, gp.). The intercellular
matrix is usually about 150 Å wide, but in patches may be as little as 50 Å. The
distance between the nuclear and cytoplasmic membranes appears to have in-
creased considerably compared with the previous stages. Much of this region of
the cytoplasm is occupied by endoplasmic reticulum. This is mainly of the granu-
lar variety and is in several forms: (1) so-called cisternae, irregular in outline and
as much as 4 \(\mu\) in length (Plate 7, fig. 30, e.r. 1); (2) circular in outline, up to $\frac{1}{4}$ \(\mu\)
in diameter (Plate 7, fig. 30, e.r. 2), and similar to the endoplasmic reticulum in
the axons (Plate 6, figs. 22, 23, 24, e.r.); (3) paired membranes (Plate 7, fig. 27,
e.r. 3, and fig. 29) which may be a stage in the formation of the Nissl substance.

Non-granular membranes of endoplasmic reticulum are also present and may
be arranged in the form described as Golgi bodies by certain authors (Sjöstrand,
1956) (Plate 7, fig. 27, e.r. 4).

The percentage of mitochondrial substance present in the cytoplasm of the
neural tube (i.e. axons, cell-bodies, and spongióblasts) was estimated (Table 1
and Text-fig. 2) and was found to be significantly less than for the preceding
stages. It is possible, however, that this is due more to an increase in the amount
of cytoplasm than to an actual decrease in the amount of mitochondrial sub-
stance.

**DISCUSSION**

Embryonic cells are constantly subjected to influences from neighbouring
cells, and in some cases (e.g. during neural induction) these influences have a
profound effect upon differentiation. For this reason the discussion will be
divided into two main sections: (1) morphological relationships between cells,
and (2) changes within the cells.
Morphological relationships between cells

(a) At the time of neural induction

In the chick embryo the developing chorda-mesoderm is believed to be responsible for inducing neural tissue (with the possible exception of the forebrain). The evidence, which comes mainly from grafting experiments, is discussed by Waddington (1952). There are several reasons for believing that neural induction starts when the head process is beginning to develop, that is, at the definitive primitive-streak stage (stage 4 of Hamburger & Hamilton, 1951). First, it is unlikely to have taken place at the previous stage (long primitive-streak stage of Waddington, 1932), for at that time the presumptive neural plate is not yet underlain by head-process mesoderm (Pasteels, 1937), and, furthermore, the whole region is capable of extensive regulation (Abercrombie, 1950; Abercrombie & Bellairs, 1954). Secondly, neural induction has probably taken place by the time the head process is well formed, for by then the regulatory powers of the presumptive neural plate have almost entirely disappeared (Abercrombie, 1950; Abercrombie & Bellairs, 1959). It seems probable, therefore, that neural induction occurs between the two stages, that is, at the definitive primitive-streak stage when the head-process mesoderm has migrated forward beneath the presumptive neural plate (Spratt, 1952).

The present results show that at the time when neural induction is believed to be taking place the lowest cells of the ectoderm and the uppermost cells of the mesoderm are separated from one another by an intercellular matrix. In no section were the cell membranes ever found in complete contact. It must be admitted, however, that closer contact might occur for brief periods only and so be overlooked. There is, indeed, some experimental evidence that in amphibians the time needed for an induction to occur is as little as 5 minutes (Nieuwkoop, 1955).

Eakin & Lehmann (1957) reported having seen in electron micrographs of amphibian gastrulae, at the time of neural induction, regions between the ectoderm and mesoderm where the cell membranes appeared to have broken down. They suggested that these might be regions of anastomosis, but, as they themselves pointed out, higher magnifications and different fixatives will be necessary before this interpretation can be accepted.

The intercellular matrix found between the ectoderm and mesoderm in the present investigation appears to be derived mainly from the basement membrane of the ectoderm. The evidence is as follows. At low electron magnifications a dense line can be seen lying parallel with the free edge of the cells at the base of the ectoderm or neural tube (Plate 1, fig. 5). This was found at all stages examined except at the time when induction was believed to be in progress (Plate 2, fig. 8). This dense line has also been reported by Duncan (1957) in this region. At higher electron magnifications, however, it can be seen that the dense line is not in fact part of the cell membrane, but that it consists of a
fine granular material (Plate 1, fig. 3). Furthermore, it is separated from the cell membrane by a similar, though less dense, region of granulation. These findings are in agreement with those of Robertson (1957) on the structure of the free surfaces of nerve- and muscle-fibres. He has also shown that this granular material is present along the base of a large number of different cell types which are known from light microscopy to possess a basement membrane. He has concluded, therefore, that the granular material is in fact the basement membrane.

TEXT-FIG. 4. Diagram to illustrate the relationship between ectoderm and mesoderm during neural induction. A, basal edge of two ectodermal cells of the presumptive neural plate prior to neural induction (see Text-fig. 1 A, D). ect., ectodermal cell; e.c.m., cell membrane of ectodermal cell; b.m., ‘basement membrane’, composed largely of granular material. B, basal edge of the same cells when they have become underlain by mesoderm, that is during neural induction (see Text-fig. 1 B, C, E). mes., mesodermal cell; m.c.m., cell membrane of mesodermal cell; b.m. (i.c.m.), the former basement membrane of the ectodermal cells which has now become pressed into an intercellular matrix between the ectoderm and mesoderm cells.

This granular material of the basement membrane is present along the base of the presumptive neural plate ectoderm before the time of induction (Plate 1, fig. 4). It is suggested, therefore, that it contributes to the intercellular matrix which lies between the ectoderm and mesoderm cells at the time of induction. It appears to be compressed during this period, for whereas the fine granular material along the free edge of the ectoderm cells was usually about 400–600 Å wide, the intercellular matrix between the ectoderm and the mesoderm cells was sometimes found to be as little as 200 Å wide (see Text-fig. 4).

(b) During neuroblast formation

When the embryo is between the stages of 16 and 21 (about 50 hours to 3½ days) many of the neuroblasts have entered the ‘grey matter’ and are beginning to differentiate. It has not been possible to recognize the developing glial cells at this stage, either by light or electron microscopy.

In most regions of the neural tube the cells are separated by an intercellular matrix of 100–150 Å in width. In some regions in the chick spinal cord, however, the intercellular matrix is considerably reduced, the two adjacent membranes
appearing to be almost in contact. It is suggested that changes at the surface between adjacent cells might serve to anchor these cells, which are probably neuroblasts, and prevent them from migrating into the ‘white matter’. This possibility is supported by the fact that anchoring is known to occur in amphibian neuroblasts growing in tissue culture, the posterior end becoming attached to the substrate while the free anterior end continues to move forward (Holtfreter, 1947). This possibility that membranes might become anchored to one another by a reduction of the intercellular matrix was suggested by Robertson (1958).

(2) Changes within the cells
(a) Intra-cellular yolk drops
The structure and fate of the intra-cellular yolk drops has been discussed in a previous paper (Bellairs, 1958). By the stage of about 48 hours most of the yolk drops have disappeared from the neural tube; this corresponds with the time at which the blood has begun to circulate and at which partially digested yolk is being brought via the vitelline veins from the yolk sac (Schechtman, 1956). Some of the fatty type of yolk drops (type B) remain, however, and have been seen as late as stage 16 (see also fig. 6 of Duncan, 1957).

It has been shown that various structural changes occur in the mitochondria as the neuroblasts develop. These are: (1) a change from circular or oval to more elongated outlines; (2) a reduction in the disruption of the cristae; and (3) an increase in electron opacity after fixation in osmium tetroxide. It is possible that these changes may reflect alterations in the physiological activity in the nervous system. Indeed, Moog (1952) has shown that during this period there is an alteration in the relative activity of various enzymes in the embryo, some of which (e.g. cytochrome oxidase) are known to be associated with mitochondria (Gustafson, 1954).

(b) Endoplasmic reticulum and Nissl substance
The characteristic feature of all neurones is the presence in their cytoplasm of Nissl substance. Palay & Palade (1955) have equated the Nissl substance with endoplasmic reticulum of the type which possesses granules attached to its membranes. The present paper provides the following evidence in favour of Palay & Palade’s interpretation. (1) There is a rise in the percentage of endoplasmic reticulum in the cytoplasm of the developing neuroblasts during the stages examined (Text-fig. 2). The greatest increase is between the 4th and 10th days (about stages 23–36) which is the time when the Nissl substance is known to be developing (Hughes, 1955). (2) It is typical of Nissl substance to be arranged around the nucleus, and it is in this region that the increase in endoplasmic reticulum chiefly occurs (cf. Plate 7, fig. 27 and Plate 5, fig. 19). (3) Although the disposition of the membranes of endoplasmic reticulum into the parallel sheets or lamellae which Palay & Palade described as typical for Nissl substance was
seldom seen in my specimens, certain configurations (Plate 7, fig. 27, e.r. 3, and Plate 7, fig. 29) which may be stages in the formation of Nissl substance became

**TEXT-FIG. 5.** Interpretative diagram of a neurone of a chick embryo at about 10 days' incubation. The whole cell is cut transversely but the axon is also cut through vertically and its terminal connexion is indicated by broken arrows. The intercellular matrix between the cell-body and the neighbouring cells is reduced in small patches; this may help to anchor the cell-body in the grey matter. Endoplasmic reticulum covered with osmiophilic granules forms the Nissl substance in the cell-body. Tubes of endoplasmic reticulum run along the axon. (In any section of this sort only a few of the tubes will be exposed along their whole length.) Water droplets (Hughes's 'vacuoles') are taken in at the tip of the axon and may pass up the tubes of endoplasmic reticulum to the cell-body (see Discussion). *H.v.*, Hughes's 'vacuoles'; *i.c.m.*, intercellular matrix; *r.i.c.m.*, reduced intercellular matrix; *m.*, mitochondrion; *N.*, developing Nissl substance; *n.*, nucleus. The neurofilaments are not shown.

prominent about the 10th day. Similarly, the basophilia of these cells as seen by light microscopy was not conspicuous until about the 9th to 10th days.

As the Nissl substance forms there is a great increase in cell volume (Hydén, 1943). There is evidence (see below) that this may be brought about, at least in part, by new material entering the neurone at the tip of the axon and passing up
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to the cell-body, possibly via tubes of endoplasmic reticulum (see Text-fig. 5). The nucleus plays a large part in the formation of proteins in the nerve cell-body (Hyden, 1943). It is possible, therefore, that the Nissl substance or its precursor is formed by the action of the nucleus on this new material. The evidence that new material may enter the cell-body in this way is as follows:

(1) Droplets of culture medium are taken in at the tips of axons growing in tissue culture. This was shown in films taken by Hughes (1953), whose material was from chick spinal ganglia of the same stage of development (7–12 days). He has called these droplets ‘vacuoles’. Hughes found that in some cases the ‘vacuoles’ passed up the axon to the cell-body.

(2) The circular structures which I have seen in the axons (Plate 6, figs. 22, 23, 24) are of comparable size (up to $\frac{1}{2} \mu$) to Hughes’s ‘vacuoles’ (up to $\frac{2}{3} \mu$). They are usually of the same shape (round) although occasionally they are more elongated. It is concluded that they may be the same structures as Hughes’s ‘vacuoles’. Similar, though smaller, structures have however recently been seen in the dendrites of adult neurones (Gray, unpublished).

(3) In some favourable sections these ‘vacuoles’, which lie one behind the other in the axons, appear to be connected by a narrow stalk (Plate 6, fig. 24). Tubes of the endoplasmic reticulum have also been shown in the present investigation to run along the axons. In places these tubes are swollen (Plate 6, fig. 26, sw) and it is suggested that these swellings may be caused by Hughes’s ‘vacuoles’ lying in the tube. Similar tubes with smaller swellings of the same type have also been reported in adult neurones (Palay, 1956, 1957).

A little granular endoplasmic reticulum is present in the cells from at least as early as the primitive-streak stage (Plate 1, fig. 2), and it seems reasonable to suppose that this might act as a pattern for the building up of similar material. There is also the possibility tentatively suggested by Palay & Palade (1955) that the non-granular endoplasmic reticulum might give rise to the granular.

There can be little doubt that many of the axons have already made their terminal connexions by the stage of 9 days’ incubation, for spontaneous activity occurs in chick embryos from the 5th day or earlier (Windle & Orr, 1934; Kuo, 1932). It is possible, therefore, that the contents of Hughes’s ‘vacuoles’ vary according to the axon connexions, and this factor could lead to variations in the differentiation of the cell-bodies. This might account to some extent for the well-known effect that terminal connexions have on the development of the cell-body (Hamburger & Levi-Montalcini, 1950; Hughes & Tschumi, 1957, 1958).

No evidence is yet available to show whether or not the tubes of endoplasmic reticulum found in each axon are continuous with the cell membrane at the tip of the axon. This possibility has been suggested for other types of cells (Palade, 1956). A discussion of the various concepts of how pinocytosis takes place (for example, see Bennett, 1956) will be more appropriate when this information has been obtained.
The nature of the neurofibrillae

Different authors have applied the term neurofibrillae to a variety of structures (see Hughes, 1954). A suitable definition might be that they are long, apparently continuous threads visible by light microscopy after silver staining. It is generally assumed that the silver acts by becoming deposited on the surfaces of membranes or similar structures. It is suggested as a result of the present investigation that there are two sets of structures in the embryonic neurone on which silver could become deposited.

1. The neurofilaments (axon filaments). These structures are about 100 Å in diameter (Plate 4, fig. 16, n.f.). They have been seen in electron micrographs soon after the neurofibrillae first became stainable with silver (stage 16). They have not been seen before this stage, although it is possible that a sufficiently wide search has not been made. They are present in adult neurones (Fernández-Morán, 1952; Palay & Palade, 1955) and correspond in size to similar structures recorded in living material by polarized light (Bear, Schmitt, & Young, 1937). For this reason it is considered that they are not fixation artefacts. It has been suggested that although individual filaments are too small to be resolved by ordinary light microscopy, aggregates of them encrusted with silver would be visible (Fernández-Morán, 1952; Palay & Palade, 1955). This is supported by the fact that in some specimens in the present investigation, ill-defined dark bands are visible which are between 150 and 300 Å wide. That is, they are too big to be single neurofilaments (which are about 100 Å wide) and too small to be tubes of the endoplasmic reticulum (which are about 300 to 500 Å wide). It is suggested that they are neurofilaments which have become clumped together, perhaps through over-fixation. Similarly, Couteaux (1956) found that the neurofilaments in the nerves of Hirudo medicinalis could be made to clump together by altering the fixative.

2. Tubes of endoplasmic reticulum. I have shown in this investigation that tubes of endoplasmic reticulum are present in the axons. They are of comparable width (up to about \( \frac{1}{2} \mu \)) to the neurofibrillae in silver preparations of chick embryos of the same stage of development. Moreover, they appear to run continuously along the axons in the same way as the neurofibrillae. Endoplasmic reticulum is also plentiful in the cell-body at this stage. Its structure as seen in sections has been described above. It is possible, however, that if a reconstruction of serial sections were made, these apparently separate units of endoplasmic reticulum would be found to be continuous with one another. Thus a continuous system of paired membranes and tubes would be present. It is suggested that this would provide a second set of surfaces upon which silver might be deposited during silver staining. Basic dyes, however, would probably stain the ribonucleoprotein granules attached to these membranes (Palay & Palade, 1955). In some photographs the walls of the endoplasmic reticulum appear to be linked by a narrower region about 75 to 100 Å wide (Plate 4, fig 17, arrow). It is possible that
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this narrow part is the edge of the wall of a connecting piece of endoplasmic reticulum which has been merely grazed by the sectioning. Alternatively, it is possible that the narrow regions are neurofilaments and that the endoplasmic reticulum and the neurofilaments are different phases of the same structure. In favour of the second suggestion is the fact that in *H. medicinalis* the neurofilaments, which are larger than in vertebrates (200 Å wide as opposed to about 100 Å), appear to have a tubular structure (see Couteaux, 1956, figs. 3 and 5).

SUMMARY

1. Developing neural tissue has been studied by electron microscopy in 73 chick embryos ranging from the primitive-streak stage to 13 days of incubation.

2. At the time of neural induction (definitive primitive-streak stage) an intercellular matrix between 200 Å and 1,000 Å wide was always found between the ectoderm of the presumptive neural plate and the underlying mesoderm. Small particles, each about 100 to 150 Å in diameter, could be seen in this matrix. Evidence is presented to show that this intercellular matrix is derived, at least in part, from the basement membrane which lies along the basal edge of the ectoderm in the preceding stage.

3. From about 3½ to 4 days' incubation patches have been seen around the base of developing axons in which the intercellular matrix is reduced in width. It is suggested that this serves to anchor the neuroblast cell-body in the mantle layer.

4. The developing neuroblasts possess tubes of endoplasmic reticulum running along their axons. Structures which are interpreted as swellings of these endoplasmic reticulum tubes can be seen in the axons between the 7th and the 13th days. It is suggested that the tubes may form a channel up which the 'vacuoles' taken in by pinocytosis (previously described by Hughes, 1953) pass to the cell-body.

5. An adaptation of Chalkley's method (1943) has been used for estimating the amounts of the endoplasmic reticulum, mitochondria, yolk drops, and other cytoplasmic constituents in relation to the cytoplasm. By this method it has been shown that there is an increase in the proportion of endoplasmic reticulum in the cytoplasm as the neuroblasts develop. This is attributed to the formation of tubes in the axon, and to the development of Nissl substance in the cell-body.

6. The proportion of the mitochondria in the cytoplasm becomes reduced between the 4th and the 10th days, as the proportion of endoplasmic reticulum rises.

ACKNOWLEDGEMENTS

I wish to thank Professor J. Z. Young, F.R.S., in whose department this work was carried out, and Dr. J. D. Robertson for the interest which they have taken in it and for their helpful criticisms. I am also indebted to Mr. N. W. Please for his advice on statistical methods. In addition I wish to express my gratitude to
Mrs. Rose Wheeler, Mr. Alan Aldrich, and Mr. Brian Cowley for their skilful technical assistance. Financial help in publishing the plates was received from the Royal Society.

REFERENCES


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EXPLANATION OF PLATES

PLATE 1

**Fig. 1.** Transverse section through presumptive neural plate ectoderm at the long primitive-streak stage (stage 3j) (see Text-fig. 1 a, d). A cell on the left stretches from the top to the bottom of the epithelium. Two endoderm cells (en.) are at the bottom of the picture. n., nucleus; A, B, and C, three types of yolk drops; c.m., cell membrane. Osmic acid fixation. Magnification ×4,700.

**Fig. 2.** Section through an ectoderm cell in the presumptive neural plate at the long primitive-streak stage (stage 3j), g.e.r., membranes of endoplasmic reticulum with granules attached to them; gran., granules about 100 Å in diameter in the cytoplasm. Osmic acid fixation. Magnification ×54,000.

**Fig. 3.** Section through the ventro-lateral part of the neural tube in the cervical region of an embryo at stage 21. The free basal edge only is shown. cyt., cytoplasm of the cell; c.m., cell membrane; d.l., dense line parallel with the cell membrane; l.z., light zone. Potassium permanganate fixation. Magnification ×130,000.
Fig. 4. Section through the base of the neural plate ectoderm at the long primitive-streak stage (stage 3, see Text-fig. 1 A, D). Granules, each about 100 to 150 Å, are attached to and perhaps intermingled with the fine granular material seen in Fig. 3. cyt., cytoplasm of the cell; c.m., cell membrane; d.L, dense line; gran., granules. Osmic acid fixation. Magnification ×111,000.

Fig. 5. Section through the ventro-lateral part of the neural tube in the anterior trunk region of an embryo at stage 12. The basal edge of the epithelium is on the right. d.L, dense line parallel with the cell membrane; c.m., cell membrane; f.m., free material between neural plate and mesoderm; m., mitochondrion. Osmic acid fixation. Magnification ×16,000.

Plate 2

Fig. 6. Transverse section through the presumptive neural plate ectoderm at the earliest head-process stage (stage 4; see Text-fig. 1 B, C, E). This electron micrograph is part of a montage. At the bottom of the picture a mesoderm cell is pressed against two ectoderm cells; the region of contact is indicated by arrows. A, type A yolk drop; B, type B yolk drop; n.e., nucleus of ectoderm cell; n.m., nucleus of mesoderm cell; e.r., endoplasmic reticulum. Osmic acid fixation. Magnification ×4,700.

Fig. 7. Endoplasmic reticulum (e.r.) consisting of elongated paired membranes, present at the head-process stage (stage 5). B, type B yolk drop with the typical empty appearance after this fixative. Potassium permanganate fixation. Magnification ×13,800.

Fig. 8. Region of 'contact' between the ectoderm and the underlying mesoderm at the earliest head-process stage (stage 4). This region is comparable to that in Fig. 6. e., ectoderm cell; mes., mesoderm cell; n.m., nucleus of mesoderm cell; i.c.m., intercellular matrix; e.r., endoplasmic reticulum; arrows indicate the cell membranes of the ectoderm and mesoderm cells respectively. Osmic acid fixation. Magnification ×30,800.

Fig. 9. Enlargement of region of 'contact' similar to the one in Fig. 8 to show granules (gr.) in the intercellular matrix. Arrows indicate the cell membranes of the ectoderm and mesoderm cells respectively. e., ectoderm cell; m., mesoderm cell. Osmic acid fixation. Magnification ×46,200.

Plate 3

Fig. 10. Ependymal edge of the hind-brain region at stage 12. I., lumen of tube containing granular material; B, type B yolk drop. Osmic acid fixation. Magnification ×3,500.

Fig. 11. Ependymal edge of neural tube in the cervical region (stage 17). This picture is part of a montage across the whole width of the neural tube. Arrows show where the two sides of the lumen have come into contact with each other in the midline. ep., ependymal cells; m.e.r., paired membranes of endoplasmic reticulum; m., mitochondrion; I., remains of lumen. Potassium permanganate fixation. Magnification ×11,500.

Figs. 12 and 13. Junction of the cell-bodies of two cells at stage 21. Cell membranes of adjacent cells are indicated by arrows. i.c.m., intercellular matrix. In Fig. 13 the intercellular matrix is present only at the top of the picture; in the lower part of the picture it is reduced or absent, the two membranes being pressed together. Potassium permanganate fixation. Magnification ×46,200.

Plate 4

Fig. 14. Section through the ventro-lateral part of the neural tube in the anterior trunk region of an embryo at stage 11. This photograph, which is part of a montage, shows a process (c.p.) extending from a cell which is probably a neuroblast. The cell process possesses several paired membranes of the endoplasmic reticulum running along it (m.e.r.) (see text). The main part of the cell is not shown but lies beyond the top of the page. Potassium permanganate fixation. Magnification ×11,500.

Fig. 15. Membranes of endoplasmic reticulum with granules attached (e.r.) from a cell in the anterior trunk region of the neural tube at stage 18. Osmic acid fixation. Magnification ×30,500.

Fig. 16. Section through the ventro-lateral part of the neural tube in the cervical region of an
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Plate 3
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Plate 5
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Plate 7
embryo at stage 36. In the lower part of the picture the section passes longitudinally through an
axon. n.f., neurofilaments; m., mitochondrion. Potassium permanganate fixation. Magnification × 38,500.

Fig. 17. Endoplasmic reticulum in the cell-body of a neuron from an embryo at stage 34. Two
profiles of endoplasmic reticulum (e.r.) are joined by a narrower region (arrow). Potassium permanganate fixation. Magnification × 38,500.

PLATE 5

Fig. 18. Longitudinal section of an axon from a neuroblast (cervical region, stage 17). m.e.r.,
paired membranes of endoplasmic reticulum; c.e.r., circles of endoplasmic reticulum; m., mito-

Fig. 19. Perihernal edge of neural tube in the cervical region at stage 16. An elongated process
extends to the edge of the neural tube (bottom left) from a cell whose nucleus lies at the top
right-hand corner of the photograph. n.m., nuclear membrane; c.m., cell membrane; e.r., mem-
branes of endoplasmic reticulum; m., mitochondrion. Potassium permanganate fixation. Magni-
fication × 12,650.

Fig. 20. The edges of two adjacent cells (stage 20) in the cervical region of the neural tube. They
are separated in places by small villous processes of other cell(s). v., villous process cut in trans-
verse section; arrows indicate the cell membranes of the two adjacent cells; n.m., nuclear mem-

Fig. 21. Meshwork of neurofilaments (n.f.) present in the cell process of a neuroblast at stage

PLATE 6

Figs. 22, 23, and 24. Longitudinal sections of axons from the white matter of the cervical
region at stage 35. ax., axon; d.b., dark bands; m., mitochondrion; e.r., endoplasmic reticulum.
Osmic acid fixation. Magnification × 26,000.

Fig. 25. Transverse sections across the white matter from the cervical region at stage 36. ax.,
axon; e.r., small circles of endoplasmic reticulum in each axon; they are tubes cut across (cf.
Fig. 26). Potassium permanganate fixation. Magnification × 30,800.

Fig. 26. Longitudinal section of an axon to show paired membranes of endoplasmic reticulum
(indicated by arrows). They are swollen in places (sw.). Potassium permanganate fixation. Magni-
fication × 38,500.

PLATE 7

Fig. 27. Transverse section across the ‘grey matter’ in the cervical region at stage 36. n.,
nucleus; c.m., cell membrane; e.r. 1, e.r. 3, and e.r. 4 are different types of endoplasmic reticulum
(see text); gp., gap between axons. Potassium permanganate fixation. Magnification × 6,900.

Fig. 28. Transverse section across a glial cell (n.g.) in the ‘white matter’ in the cervical region
at stage 36. Note the sparseness of endoplasmic reticulum in this cell. The borders of the cell are
indicated by arrows. Around the glial cell are many axons cut in transverse section, and in each
of these, transverse sections of endoplasmic reticulum can be seen as small dots (e.r.). Potassium
permanganate fixation. Magnification × 5,750.

Fig. 29. Endoplasmic reticulum in the cell-body of a neuron (stage 35) arranged as paired

Fig. 30. Part of the cell-body of a neuron (cervical region, stage 27), e.r. 1 and e.r. 2, endo-
plasmic reticulum (see text). Osmic acid fixation. Magnification × 19,250.

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